## Amendments to the Specification

At **page 8, line 29**, please add the following paragraphs:

--Figure 18. Expanded region of the 1D $^1$H WaterLOGSY spectrum of a 10 compound mixture (see text) in the presence of the protein cdk2 recorded with a 40 Hz and 2 s long RF presaturation field applied off-resonance (a) and at the $H_2O$ chemical shift (b). (c) Difference spectrum obtained by subtracting spectrum (b) from spectrum (a). Human cdk2 protein was expressed in Sf9 insect cells using a recombinant baculovirus encoding cdk2. The NMR sample was in Phosphate Buffered Saline (PBS) (8% $D_2O$) and the protein concentration was 10 μm. The spectra have been recorded at Te = 19° C with a Varian Inova 600 MHz spectrometer. The $H_2O$ solvent suppression was achieved with the $H_2O$ excitation sculpting sequence (Hwang et al., *J. Magn. Reson. A112*:275-279 (1995). A total of 256 scans were recorded for each spectrum (a,b). The chemical structures of the two molecules are depicted. Positive and negative signals in (c) identify cdk2 interacting and not interacting molecules, respectively.

C1

Figure 19. One-dimensional reference (upper) and WaterLOGSY with NOE-ePHOGSY (lower) spectra recorded for the 10-compound chemical mixture in the presence of 10 μm cdk2. The WaterLOGSY and the reference spectra were recorded at Te = 17° C with 256 and 128 scans, respectively. The $H_2O$ solvent suppression in both experiments was achieved with the $H_2O$ excitation sculpting sequence (Hwang et al., *J. Magn. Reson. A112*:275-279 (1995). The WaterLOGSY was recorded with a 38 ms long 180° $H_2O$ selective Gaussian pulse. This pulse can be set also to only 10 to 20 ms length, because no high selectivity is required. The relaxation and mixing times were 2.6 and 2 s, respectively. Positive and negative signals in the lower spectrum identify cdk2 interacting and not interacting molecules, respectively. The asterisk indicates the methyl group resonances of the cdk2 ligand ethyl alpha-(ethoxycarbonyl)-3-indoleacrylate.

Figure 20. Expanded region of 1D WaterLOGSY with NOE-ePHOGSY (lower) and ROE-ePHOGSY (upper) spectra for the 10-compound mixture in the presence of 10 μm cdk2.

The spectra were recorded at Te = 17° C with 1024 scans and with 2.6 s relaxation delay. The mixing and spin-lock times were 2 and 0.3 s, respectively. The signal at 4.06 ppm, labeled with an asterisk, originates from an exchangeable proton resonance.

C1 cont

Figure 21. Expanded region of the WaterLOGSY $^1$H 2D PFG DQ spectra of the 10-compound mixture with cdk2. The spectra above and below were obtained respectively by subtracting and adding the two spectra recorded with $H_2O$ and an off-resonance presaturation rf field of 40 Hz and length 2 s. The 45°/135° version of the experiment was recorded at Te = 19° C with pulsed field gradients tilted at the magic angle for better solvent suppression. The excitation DQ period was 41 ms long and 16 scans were recorded for each of the 128 $t_1$ increments. In the difference spectrum the cross peaks of the two $CH_3$-$CH_2$ moieties (labeled A) of the cdk2 ligand ethyl alpha-(ethoxycarbonyl)-3-indoleacrylate have opposite sign when compared to the cross peaks of the $CH_2$-$CH_2$ moiety (labeled B) of mono-methyl succinate.--

---

At **page 14, line 5**, please add the following paragraphs:

---

C2

--Water plays a pivotal role in the protein-ligand, protein-protein, and protein-DNA recognition mechanisms. Based on numerous observations in this regard (Otting, *Progr. NMR Spectrosc., 31*:259-285 (1997); Dalvit et al., *J. Biomol. NMR, 13*:43-50 (1999); Otting et al., *Science, 254*(5034):974-980 (1991); Otting et al., *J. Am. Chem. Soc., 111*:1871-1875 (1989); and Kallen et al., *J. Mol. Biol., 292*:1-9 (1999)), $H_2O$ can be used for the detection of molecules interacting with a protein. Two different classes of experiments can be used for this purpose, i.e., a steady state NOE experiment with on-resonance saturation applied at the water chemical shift or a NOE experiment with selective inversion of the $H_2O$ signal and with a long mixing time. Numerous schemes have been devised for selective water excitation (Otting, *Progr. NMR Spectrosc., 31*:259-285 (1997) and references therein). A member of this type of experiments is the NOE-ePHOGSY and related experiments (Dalvit et al., *J. Magn. Reson. B., 109*:334-338 (1995); Dalvit, *J. Magn. Reson. B., 112*(3):282-288 (1996); Melacini et al., *J. Biomol. NMR, 13*:67–71 (1999a); Melacini et al., *J. Biomol. NMR, 15*:189-201 (1999b).

The saturation of water yields the following effects: (i) saturation of some of the αH protein resonances, (ii) complete saturation of the fast exchanging NH and OH protons of the protein and small molecules resonating at the $H_2O$ chemical shift, (iii) partial or total saturation of rapidly exchanging NH and OH protons of the protein and small molecules resonating at a chemical shift different from $H_2O$, (iv) magnetization transfer from bulk water to bound water located in different cavities of the protein, and (v) magnetization transfer from bulk water to the squeezed water at the protein–ligand interface. Inversion of most of this magnetization is achieved in the NOE-ePHOGSY experiment with the exception, in large biomolecules, of the αH protein signals resonating at the $H_2O$ chemical shift (i). The acquisition of these experiments is technically demanding when working in $H_2O$. Often the effects observed in the difference spectra are very small. Radiation damping and demagnetizing field mechanisms originating from bulk water can introduce artifacts and mask the small effects (Sobol et al., *J Magn Reson.*, *130*(2):262-271 (1998); Price, *Annual Reports on NMR Spectroscopy* (Ed., Webb, A.), Academic Press, New York, vol. 38, pp. 289-354 (1999)). However, it is possible to overcome these problems by properly using pulsed field gradients.

C2 cont

WaterLOGSY (Water-**L**igand **O**bservation with **G**radient **S**pectrosco**Y**) is the term applied for these experiments used for detection of ligands via bulk water. Figure 18 shows the principle of the experiment recorded with steady state NOE applied to a mixture of 10 low molecular weight compounds (concentration 100 μm) in the presence of 10 μm of cyclin-dependent kinase 2 (cdk2) protein (Mw ~ 34 kDa). The molecules of the mixture are 3-methylenecyclopropane-trans-1,2-dicarboxylic acid, mono-methyl succinate, s-benzylthioglycolic acid, 3,3-dimethylacrylic acid, 1,2,4-triazole, 5,5-dimethyl-2-4-oxazolidinedione, 2,2-dimethyl-1,3-dioxane- 4,6-dione, fluoroacetamide, pinacolone and ethyl alpha-(ethoxycarbonyl)-3-indoleacrylate. The expanded region contains only the two methyl group signals (1.29 and 1.25 ppm) of the indole derivative and the methyl t-butyl signal (1.08 ppm) of pinacolone. The spectra in (a) and (b) were recorded with water and off-resonance saturation, respectively. A weak positive NOE effect (negative signal) for pinacolone and a weak negative NOE effect (positive signals) for the indole derivative are

observed in the difference spectrum (Figure 18c). Pinacolone does not interact with the protein and therefore displays a positive NOE with $H_2O$ whereas the indole derivative that interacts with the protein (measured Ki is in the high μm range) displays a negative NOE stemming from the effects associated to the saturation of bulk $H_2O$, as described above.

The 1D WaterLOGSY experiments with the $H_2O$ presaturation scheme can give rise to small artifacts originating from the difference spectroscopy method. However, the version with the NOE-ePHOGSY scheme is completely devoid of artifacts. Even very weak effects can be analyzed with confidence. This can be appreciated in Figure 19. The 10 small molecules mixture contains NMR signals consisting mostly of sharp singlets. Comparison of the 1D NOE-ePHOGSY (lower spectrum) with the 1D reference spectrum (upper spectrum) allows easy identification of the only molecule interacting with the protein. The measuring time of the WaterLOGSY spectrum of Figure 19 was only 20 min. The quality of the spectra obtainable with the NOE-ePHOGSY scheme and the sensitivity of the experiment have allowed application of the method to protein concentrations as low as a few hundred nM (data not shown). The exchangeable proton resonances, when visible, will also appear as positive peaks in the WaterLOGSY experiments. These peaks usually can be easily recognized in the spectrum. However, if doubts remain it is sufficient to record the WaterLOGSY experiment with the ROE-ePHOGSY scheme for the unambiguous identification of the exchangeable resonances. Figure 20 shows application of this strategy. The positive peak at 4.06 ppm observed in the WaterLOGSY with NOE step (lower spectrum) does not originate from a ligand of cdk2, but it is simply an exchangeable proton resonance as confirmed by the WaterLOGSY experiment with ROE step (upper spectrum).

The WaterLOGSY schemes (either with $H_2O$ presaturation or NOE-ePHOGSY) can be also used in 2D experiments (DQ, TOCSY, etc.). Use of Water- LOGSY in the $^1H$ 2D PFG DQ experiment applied to our compound mixture is shown in Figure 21. The signals of the $CH_3$-$CH_2$ moiety of the cdk2 ligand ethyl alpha-(ethoxycarbonyl)-3-indoleacrylate and the signals of the $CH_2$-$CH_2$ moiety of mono methyl succinate are visible in this expanded spectral region (lower spectrum). These signals are also visible in the DQ difference spectrum (upper spectrum).

However, the signals of the ligand are easily recognized because they have opposite sign when compared to the signals of mono methyl succinate.

C2 cont

Thus, it is possible to use the large reservoir of bulk $H_2O$ magnetization to detect via different transfer mechanisms small molecules that interact with a target biomolecule (proteins, DNA or RNA fragments). The method, like all the techniques based on ligand resonance observation, has the disadvantage that it does not provide information about the ligand binding site. Despite this drawback the technique represents a rapid means for ligand identification.--

Please replace the paragraph beginning at **page 14, line 5** (as originally filed), with the following amended paragraph.

C3

Since the WaterLOGSY experiment relies on the transfer of magnetization from bulk water to detect the binding interaction, it is a very sensitive technique. As such, the concentration of target molecule (e.g., protein) in each sample preferably can be reduced to no greater than about 10 μM (preferably, about 1 μM to about 10 μM) while the concentration of each compound can be about 100 μM. This results in ratios of ~~target molecule to compounds~~ test compound to target molecule in each sample reservoir of about 100:1 to about 10:1. The exact concentrations and ratios used can vary depending on the size of the target molecule, the amount of target molecule available, the desired binding affinity detection limit, and the desired speed of data collection. In contrast to the relaxation-editing method, there is no need to collect a comparison or control spectrum to identify binding compounds from nonbinders. Instead, binding compounds are distinguished from nonbinders by the opposite sign of their water-ligand nuclear Overhauser effects (NOEs).